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Differential costs of reproduction in females and hermaphrodites in a gynodioecious plant

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• *Background and Aims* Plants exhibit a variety of reproductive systems where unisexual (females or males) morphs coexist with hermaphrodites. The maintenance of dimorphic and polymorphic reproductive systems may be problematic. For example, to coexist with hermaphrodites the females of gynodioecious species have to compensate for the lack of male function. In our study species, *Geranium sylvaticum*, a perennial gynodioecious herb, the relative seed fitness advantage of females varies significantly between years within populations as well as among populations. Differences in reproductive investment between females and hermaphrodites may lead to differences in future survival, growth and reproductive success, i.e. to differential costs of reproduction. Since females of this species produce more seeds, higher costs of reproduction in females than in hermaphrodites were expected. Due to the higher costs of reproduction, the yearly variation in reproductive output of females might be more pronounced than that of hermaphrodites.
• *Methods* Using supplemental hand-pollination of females and hermaphrodites of *G. sylvaticum* we examined if increased reproductive output leads to differential costs of reproduction in terms of survival, probability of flowering, and seed production in the following year.
• *Key Results* Experimentally increased reproductive output had differential effects on the reproduction of females and hermaphrodites. In hermaphrodites, the probability of flowering decreased significantly in the following year, whereas in females the costs were expressed in terms of decreased future seed production.
• *Conclusions* When combining the probability of flowering and seed production per plant to estimate the multiplicative change in fitness, female plants showed a 56 % and hermaphrodites showed a 39 % decrease in fitness due to experimentally increased reproduction. Therefore, in total, female plants seem to be more sensitive to the cost of reproduction in terms of seed fitness than hermaphrodites.

Key words: Gynodioecy, pollen limitation, hand-pollination, *Geranium sylvaticum*, cost of reproduction, maintenance of gynodioecy, seed production.

INTRODUCTION

In plants and animals, current reproduction may occur at the expense of future growth, survival or reproduction (e.g. Williams, 1966; Bell, 1980). In plants, evidence for such cost of reproduction has been found in several studies (e.g. Horwitz and Schemske, 1988; Zimmerman and Aide, 1989; Calvo, 1990; Karlsson *et al.*, 1990; Primack and Hall, 1990; Primack *et al.*, 1994; Aragón *et al.*, 2009), although some studies have failed to demonstrate costs of reproduction (e.g. Reekie and Bazzaz, 1987; Horwitz and Schemske, 1988; Jennersten, 1991). Costs of reproduction may also play a role in the evolution of plant reproductive systems by eliciting selection on life-history traits that are related to sexual dimorphism, such as age and size of first reproduction, frequency of reproduction and number of flowers. For example, in dioecious plant species, females generally have higher reproductive allocation than males and thus cost of reproduction is expected to be higher in females (Bawa, 1980). Indeed, several studies on dioecious species suggest lower frequency of reproduction, higher mortality and lower growth rates in females compared with males (e.g. Meagher and Antonovics, 1982; Ågren, 1988; Garcia and Antor, 1995; Antos and Allen, 1999; Nicotra, 1999; Rocheleau and Houle, 2001; Álvarez-Cansino *et al.*, 2010; for a review, see Obeso, 2002). In gynodioecious plant species in which female and hermaphroditic plants coexist, females allocate resources only for seed production, whereas hermaphrodites also allocate to pollen production. Differences in total reproductive allocation may lead to differential demographic costs of reproduction in females and hermaphrodites. Given the higher seed production of female plants observed in several gynodioecious species, it is likely that the overall resource allocation to reproduction is higher in females compared with hermaphrodites (Dawson and Geber, 1999). Due to their higher reproductive output in terms of seed production, the costs of reproduction should then be higher in females and these costs would be expressed as lower survival, growth or future reproduction.

Differential costs of reproduction may also contribute to the maintenance of gynodioecy. Ultimately, to coexist with hermaphrodites the females of gynodioecious species have to compensate for the lack of male function, and to outperform hermaphrodites in one or several aspects of female reproduction (e.g. Lewis, 1941; Lloyd, 1975; Charlesworth and

Charlesworth, 1978). In our study species, the perennial gynodioecious *Geranium sylvaticum*, female plants generally produce more seeds than hermaphrodites (Asikainen and Mutikainen, 2003). However, the relative seed fitness advantage of females varies significantly between years within populations as well as among populations (Asikainen and Mutikainen, 2003). This variation in relative seed fitness might be partly explained by differences in costs of reproduction between the sexes if these differences lead to parallel differences in temporal regulation of reproductive allocation, either within or between seasons (Ashman, 1992). Due to the putatively higher costs of reproduction, yearly variation in reproductive output of females may be more pronounced than that of hermaphrodites, especially if high reproductive output in one season decreases the probability of reproducing in the following season. Therefore, the differential costs of reproduction may confer to the temporal variation in the relative seed fitness of females and further contribute to the maintenance of females with hermaphrodites.

In this study we examined if experimentally increased reproductive output (i.e. the number of seeds produced per flower and number of seeds produced per plant) is associated with decreased survival or future reproductive success in the gynodioecious *Geranium sylvaticum*. In addition, we studied whether females and hermaphrodites behave differently in their future reproductive investment. Reproductive output was increased by hand-pollinating all flowers of female and hermaphroditic plants in three natural populations to examine especially if females and hermaphrodites express differential costs of reproduction.

MATERIALS AND METHODS

Study species and populations

Geranium sylvaticum (Geraniaceae) is a self-compatible, perennial herb with a gynodioecious breeding system (Vaarama and Jääskeläinen, 1967). It has a Eurasian distribution (Hultén and Fries, 1986). This study was conducted in Finland, where *G. sylvaticum* is common and occurs in meadows, roadsides and herb-rich forests. The flowers of *G. sylvaticum* are regular with five brightly coloured petals that vary from deep purple to white, and surround two whorls of five stamens and five pistils. The anthers of female plants are either reduced or nonfunctional. *Geranium sylvaticum* starts flowering in the beginning of June in southern Finland. Flowering lasts >3 weeks in our study populations (Asikainen and Mutikainen, 2005). *Geranium sylvaticum* is protandrous, presenting the pollen before the stigma becomes receptive. However, self-pollination by geitonogamy is possible. The flowers are visited by a variety of insects, including bumblebees, syrphid flies and other dipterans. The gynoecium of *G. sylvaticum* has five locules each containing two ovules. The fruit is a schizocarp with usually only five or fewer than five seeds developing in each fruit. The seeds mature in about 3 weeks after pollination. Just before the fruit matures, it changes from green to brown and finally the awns separate from the central axis to disperse the seeds. Our preliminary results suggest that the sex determination of *G. sylvaticum* is controlled via an interaction between nuclear and cytoplasmic genes (i.e. CMS; Asikainen 2004).

Fieldwork was conducted during summers 2001 and 2002 in southern Finland in three populations that were chosen based on their habitat and sex ratio. The first population, Katariinanlaakso, is located in a meadow in Turku (60 °23′N; 22 °19′E). The second population, Paimio, is located in a deciduous forest 27 km south-east from Turku (60 °25′N; 22 °45′E). The third population, Seili, is located in a meadow in the middle of a mixed forest on the island of Seili 29 km south-west from Turku (60 °14′N; 21 °59′E). The female frequencies in the study populations were 4·6 %, 16·5 %, and 23·0 % in 2000, respectively, and represent the range of variation previously observed in *G. sylvaticum* (Asikainen and Mutikainen, 2003). In 2002 we recorded the female frequencies of the study populations again and found them to be stable (4·4 %, 17·0 % and 26·2 %, respectively; Asikainen, 2004). In summer 2000, the sizes of the populations were 1464, 375 and 1110, respectively (Asikainen and Mutikainen, 2003).

Experimental design

In order to study whether an increase in current reproductive effort affects future survival, flowering probability, and reproductive output in *G. sylvaticum*, the reproductive effort of female and hermaphroditic plants was experimentally manipulated by pollinating all flowers within the experimental plants. In this study, the fruit set (i.e. fruits/flower) of open-pollinated females and hermaphrodites varied from as low as zero to one with an average value of 0·50 (s.e. 0·025). Given these values, it is feasible for the plants to have all of their flowers visited within a season. Therefore, the pollination treatment applied is realistic. We marked 40 female and 40 hermaphroditic haphazardly chosen flowering plants in each study population and counted the number of flower buds on each plant. The number of flowers per plant was $73{\cdot}51 \pm 5{\cdot}63$ (mean $\pm$ s.e.; there is no significant difference in flower number per plant between the genders (Asikainen and Mutikainen, 2003). In each population, the marked plants were randomly assigned to two treatments: all flowers were hand-pollinated with an excessive number of pollen grains (hand-pollination) or plants were not manipulated and were open to natural pollination (open pollination). The flowers were pollinated by applying pollen onto the receptive stigmas with an insect pin. We chose a different pollen donor for each hand-pollinated plant. The distance between the pollen recipient and the pollen donor was always at least 10 m. Each day throughout the flowering period, all new receptive flowers were pollinated. When all of the flowers had withered light mesh bags were placed around the developing fruits to collect the seeds. The seeds were collected and counted when ripe; this happened about 3 weeks after the end of flowering. In the following year, the state of the experimental plant was recorded as dead, flowering, or non-flowering. The number of flowers was counted and the seeds collected as described above when they were ripe.

Statistical analyses

To examine if hand pollination had a significant effect on reproductive output in 2001, the effects of sex, population

and treatment on seed production (i.e. the number of seeds produced per flower and number of seeds produced per plant) were analysed by three-way ANOVA. Population was treated as a fixed factor since we had only three populations that were chosen based on their sex ratios to represent the range of variation previously observed in *G. sylvaticum*. Therefore they do not represent a random sample of all available populations of *G. sylvaticum*. The number of flowers was used as covariate in the analyses of seeds produced per plant. The flowering probability in 2002 was treated as a binary variable (flowered/did not flower) and was analysed with a logistic regression. Population, treatment and gender were treated as categorical variables. The main effects and their interactions were entered into the model, and the model with best fit was chosen using stepwise backward model selection. The significance of the model was estimated using the likelihood ratio method (Hosmer and Lemeshow, 1989; Norusis, 1993). We tested whether flowering female and hermaphroditic plants in 2002 differed in seed production (seeds per flower and seeds per plant) or number of flowers produced using ANOVA with gender, population and treatment (open-pollinated/hand-pollinated in 2001) as fixed factors. To meet the assumptions of ANOVA better, seed per flower was log-normally transformed and the number of flowers per plant was square root-transformed. Since the gender of each plant was determined from a few firstly opened flowers, those plants that turned out to be intermediate or had both perfect and pistillate flowers were excluded. Further, some plants had to be excluded since they were lost or partly eaten by herbivores. Thus, the final sample sizes were unequal. A total of 185 plants (71 females and 114 hermaphrodites) were included in the statistical analyses. In 2002, 133 plants (53 females and 80 hermaphrodites) flowered. The means and standard errors presented in the figures are back-transformed values. All statistical tests were performed with SPSS statistical software (Norusis, 1993).

RESULTS

Effect of supplemental hand-pollination

Supplemental hand-pollination increased current-year seed production per flower by 29 % (i.e. open pollination 0·93 ± 0·06 (mean ± s.e.); hand-pollination 1·2 ± 0·06; Table 1). Hand-pollinated plants produced 2·1 times more seeds per plant than open-pollinated plant, although this difference was not statistically significant (Table 1). The number of seeds produced per flower and per plant also differed among the populations (Table 1). In addition, the interaction between gender and population was significant (Table 1). This significant interaction is caused by the fact that in one of the populations the genders produced equal number of seeds, whereas in the other two populations females produced substantially more seeds than the hermaphrodites (data not shown). In all, hand-pollination proved to be effective since supplemental hand-pollination increased the reproductive output of *G. sylvaticum*.

Costs of reproduction

None of the experimental plants died during the study. Hand-pollination in 2001 had differential effects on the probability of flowering in the following season in females and hermaphrodites; the interaction between gender and pollination was the only factor that significantly explained the probability of flowering in the logistic regression (change in the log likelihood $\Delta LL = 12{\cdot}566$, d.f. = 1, $P < 0{\cdot}001$; Table 2). In

TABLE 1. *Results of analyses of variance testing for differences between genders, among three populations, and between open-pollinated and hand-pollinated plants in number of seeds per flower and number of seeds per plant in* Geranium sylvaticum *in 2001 and 2002*

		Seeds/flower		Seeds/plant	
Source of variation	d.f.	MS	*P*	MS	*P*
2001					
Gender	1	0·938	<0·001	9644·91	0·063
Population	2	0·633	<0·001	9832·83	0·030
Pollination	1	0·699	0·002	6016·01	0·141
Gender × populationn	2	0·314	0·012	9778·18	0·031
Gender × pollination	1	0·170	0·117	2227·60	0·369
Population × pPollination	2	0·093	0·262	4726·10	0·182
Gender × population × pollination	2	0·070	0·363	5810·92	0·124
No. of flowers	1			1126867·29	<0·001
Error	173	0·069		2749·55	
2002					
Gender	1	0·385	0·024	2451·00	0·145
Population	2	2·666	<0·001	26453·04	<0·001
Pollination 2001	1	0·142	0·169	1241·87	0·298
Gender × population	2	0·282	0·025	1330·83	0·314
Gender × pollination 2001	1	0·644	0·004	8090·59	0·009
Population × pollination 2001	2	0·172	0·102	4854·68	0·016
Gender × population × pollination 2001	2	0·026	0·703	2260·22	0·141
No. of flowers	1			421017·68	<0·001
Error	121	0·074		1136·57	

TABLE 2. *Results of a logistic regression testing for differences between females and hermaphrodites (Gender), between open-pollinated and hand-pollinated plants (pollination) and among populations in probability of flowering in the year following the pollination treatment*

Variable	d.f.	Wald χ^2	*P*
Included in the model			
Gender × pollination	1	0·644	0·004
Variables not included in the model	d.f.	Score	*P*
Gender	1	2·217	0·136
Population	2	2·231	0·328
Pollination	2	0·614	0·433
Gender × population	2	2·267	0·263
Population × pollination	2	0·282	0·868
Gender × population × pollination	2	0·148	0·928

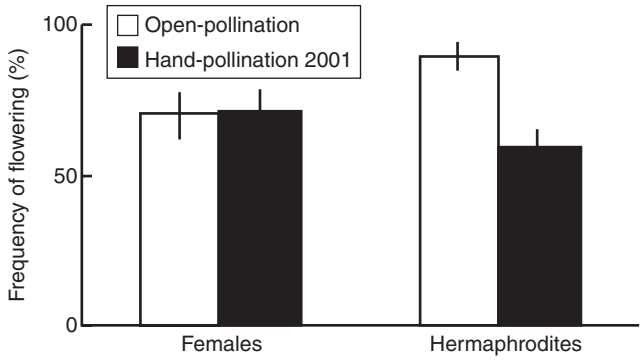

FIG. 1. Flowering frequency of females and hermaphrodites of the gynodioecious *Geranium sylvaticum* in 2002. In 2001, the plants were either hand-pollinated or left open-pollinated. Standard errors were calculated as s.e. = $\sqrt{(pq/n)}$, where p is a proportion of flowering individuals, q is a proportion of individuals that did not flower and n is a number of plants of particular gender and treatment.

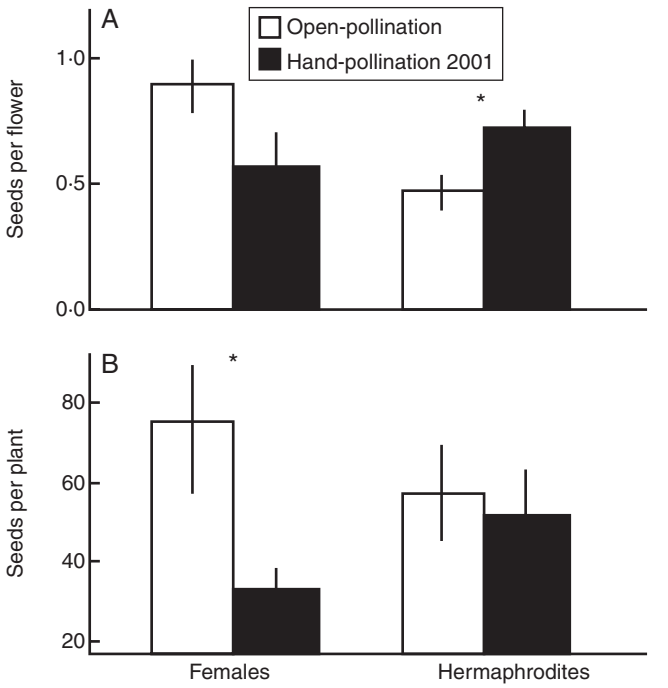

FIG. 2. (A) Number of seeds produced per flower (mean ± s.e.) and (B) number of seeds produced per plant in flowering females and hermaphrodites of the gynodioecious *Geranium sylvaticum* in 2002. In 2001, the plants were either hand-pollinated or left open-pollinated. An asterisk indicates a significant difference ($0{\cdot}01 \le P < 0{\cdot}05$) between open-pollinated and hand-pollinated plants.

females, 71·4 % of the hand-pollinated plants flowered, whereas in hermaphrodites only 58·8 % of the hand-pollinated plants flowered (Fig. 1). However, the flowering frequency of the hand-pollinated females did not differ from that of the open-pollinated females (Fig. 1). Thus, the decrease in the probability of flowering due to hand pollination was significant only in hermaphrodites (Fig. 1).

The number of flowers produced in 2002 did not differ between the treatments ($F_{1,123} = 2{\cdot}234$, $P < 0{\cdot}138$), between females and hermaphrodites ($F_{1,123} = 0{\cdot}210$, $P < 0{\cdot}648$), or among the populations ($F_{2,123} = 0{\cdot}922$, $P = 0{\cdot}400$). There were no statistically significant interactions for the number of flowers. Thus, experimentally increased reproductive output in 2001 did not affect the number of flowers per plant in the following year.

Similarly to the year of the hand pollination, we found significant differences among populations in seed production of the plants flowering in 2002, whereas supplemental hand-pollination in 2001 did not have a significant main effect on seed production in the following year (Table 1). However, there was a significant interaction between the hand-pollination treatment and plant gender for both seeds per flower and seeds per plant (Table 1 and Fig. 2). Due to this significant interaction, we tested the differences in seed production between the pollination treatments separately for females and hermaphrodites with *t*-tests. The seed production of hand-pollinated females was reduced compared with that of open-pollinated females (seeds per flower $t = -1{\cdot}743$, d.f. = 51, $P = 0{\cdot}087$; seeds per plant $t = 2{\cdot}445$, d.f. = 34·476, $P = 0{\cdot}020$; Fig. 2A, B). Seeds produced per flower of hand-pollinated hermaphrodites were significantly increased compared with those of open-pollinated hermaphrodites ($t = 2{\cdot}331$, d.f. = 78, $P = 0{\cdot}022$; Fig. 2A). However, seeds produced per plant did not differ between hand- and open-pollinated hermaphrodites ($t = 0{\cdot}272$, d.f. = 78, $P = 0{\cdot}786$; Fig. 2B). These results indicate that the costs of experimentally increased reproductive output were expressed in terms of seed production in females but not in hermaphrodites.

To estimate the total change in future seed fitness due to the supplemental hand pollination, we calculated a multiplicative seed fitness estimate for all females and all hermaphrodites, based on the mean values over all populations. We first calculated the probability of flowering in the hand-pollinated plants relative to that of the open-pollinated plants (females 0·71/0·70; hermaphrodites 0·59/0·90) and the number of seeds produced per hand-pollinated plants relative to the open-pollinated plants (females 32·4/73·6; hermaphrodites 53·1/57·6). We then multiplied the two relative fitness values to get an estimate of a multiplicative change in fitness due to the hand-pollination (females $1{\cdot}01 \times 0{\cdot}65 = 0{\cdot}44$ and hermaphrodites $0{\cdot}66 \times 0{\cdot}92 = 0{\cdot}61$). Thus, the estimated change in seed fitness due to hand pollination was −56 % in females

and −39 % in hermaphrodites. Note that the pollen production of hermaphrodites was not estimated.

DISCUSSION

Increased reproductive investment by supplemental hand pollination revealed substantial costs of reproduction in both females and hermaphrodites of *Geranium sylvaticum*. In females, the costs of reproduction were expressed as decreased individual seed production, whereas in hermaphrodites costs were expressed as a lower probability of flowering in the following year. In several other species the costs of reproduction have also been demonstrated as reduced flowering probability in the following year (Zimmerman and Aide, 1989; Primack and Hall, 1990; Karlsson *et al.*, 1990, Ågren and Willson, 1994), whereas other studies have found that experimentally increased reproductive effort reduces subsequent flower production (Klinkhamer and De Jong, 1987; Stanton *et al.*, 1987; Evans, 1991). When the total fitness effect of supplemental hand pollination was estimated, it was found that the costs of reproduction were higher in females than in hermaphrodites.

Several studies have suggested that lower frequency of reproduction, higher mortality and lower growth rates correlate with high reproductive output in females of dioecious plant species (e.g. Meagher and Antonovics, 1982; Ågren, 1988; Garcia and Antor, 1995; Antos and Allen, 1999; Leigh *et al.*, 2006) and there is experimental evidence for higher costs of reproduction in females compared with males (e.g. Nicotra, 1999; Rocheleau and Houle, 2001; Álvarez-Cansino *et al.*, 2010; for a review, see Obeso, 2002). The differential effects of experimentally modified reproductive investment on survival and fecundity of females and hermaphrodites have been studied less frequently in gynodioecious species. Corresponding to our results, Ågren and Willson (1994) found that high reproductive output reduced the probability of flowering in hermaphrodites of *G. sylvaticum* but did not affect survival or the number of flowers produced in the following year. Ågren and Willson (1994) studied the differences in costs of reproduction between two populations and between two *Geranium* species. However, we can only compare our results on the hermaphroditic plants to those of Ågren and Willson (1994) since female plants were not included in their study. Ashman (1992) found evidence for costs of reproduction both within and between flowering seasons in the gynodioecious *Sidalcea oregana* ssp. *spicata* using hand-pollinated plants and plants that were prohibited from pollination. She found that allocation to inflorescences was slightly lower in females than in hermaphrodites. In the following season, both genders responded to the manipulations by decreasing allocation to inflorescences, whereas the manipulation did not affect vegetative growth. Contrary to our results, the sexual morphs did not differ in their responses to the manipulation of the reproductive effort conducted in the previous season (Ashman, 1992).

In the present study, the open-pollinated females produced more seeds per plant than hermaphrodites. However, when the plants were hand-pollinated the previous year, the hermaphroditic plants produced more seeds than the female plants. This result is unexpected, and suggests that the cost of reproduction at the individual level is severe for the female plants. However, the population sex ratio is likely to modify the costs at the population level. The sex ratios of *G. sylvaticum* populations are highly biased; female frequencies vary from zero to about 26 % (Asikainen and Mutikainen, 2003). Therefore, even if 40 % of the hermaphrodites do not flower after a year with a high seed production (as our results suggest), there would still be significantly more flowering hermaphrodites than females in a given population after a year of high reproductive success. If these flowering hermaphrodites then produce more seeds per plant than female plants, the seed fitness of hermaphroditic plants would be significantly higher than that of females at the population level. However, although the relative seed fitness varies among the years, female plants generally produce more seeds than hermaphrodites (Asikainen and Mutikainen, 2003). Furthermore, it seems that the natural yearly variation in seed fitness of hermaphroditic plants is not as pronounced as that in female plants (see below). Therefore, the hand-pollination of all flowers might have been more 'unnatural' for hermaphrodites than for the females, and might have led to overestimation of the costs, especially in the hermaphroditic plants. In this study, the difference in costs of reproduction between females and hermaphrodites did not vary among the study populations, suggesting that the population sex ratio might be of lower relevance. However, the female frequency in our study populations varied within a rather narrow range (0–26 %). Therefore, it might not be possible to identify the potential effects of the female frequency on the population level differences in costs of reproduction between females and hermaphrodites. To our knowledge, there are no previous studies on the effects of sex ratio on costs of reproduction in gynodioecious species.

There is significant yearly variation in the relative seed fitness advantage of females in *G. sylvaticum*; on average, females produced 1·2, 1·7 and 1·3 times as many seeds as hermaphrodites in 2000, 2001 and 2002 (Asikainen and Mutikainen, 2003; Asikainen, 2004). The present results suggest that part of this yearly variation in relative seed fitness might be explained by differences in costs of reproduction between the genders. Ashman (1992) has suggested that the differences in costs of reproduction between the genders may lead to differences in the temporal regulation of reproductive allocation, either within or between seasons. We followed the reproductive success of eight populations for 3 years. In these eight populations a year with high seed production was followed by a year with a total or almost total loss of seed production. However, this phenomenon was much clearer in females than in hermaphrodites (E. Toivonen and P Mutikainen, unpubl. res.). In addition, in females, a year of low seed production was followed by a substantially better year in terms of seed production in several of the populations. Since the changes in the seed production of hermaphrodites did not correspond to the changes in seed production of females, it seems unlikely that the low seed production was caused by unfavourable environmental conditions, such as variation in pollinator abundance. Thus, it seems that yearly variation in reproductive output is more pronounced in females than in hermaphrodites. The present results suggest that the differential costs of reproduction contribute to the

temporal variation in the relative seed fitness in this species. Unfortunately, to our knowledge, the relative seed fitness of the sex morphs in other gynodioecious species has only been studied for 1 year.

Our results suggest that differences in the magnitude of costs of reproduction between females and hermaphrodites contribute to annual variation in the relative seed fitness of female plants in this gynodioecious species. Consequently, they might also contribute to the maintenance of the gynodioecious breeding system. Furthermore, if costs of reproduction in females and hermaphrodites occur in asynchrony, the relative selective advantage of each of the two sex morphs might vary annually, affecting the maintenance of the two sex morphs. However, it would be of interest to examine how large this contribution is relative to other factors, such as the frequency-dependent selection, and whether the costs of reproduction affect maintenance of gynodioecy in other species.

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